Histol Histopathol (2007) 22: 1099-1107

http://www.hh.um.es

Histology and
Histopathology
Cellular and Molecular Biology

Expression of heat shock protein 70 in renal cell carcinoma and its relation to tumor progression and prognosis

U. Ramp[1], C. Mahotka[1], S. Heikaus[1], T. Shibata[1], M.O. Grimm[2], R. Willers[3] and H.E. Gabbert[1]

[1]Institute of Pathology, [2]Department of Urology and [3]Computer Center, Heinrich-Heine University Hospital, Duesseldorf, Germany

Summary. Heat shock proteins (HSPs) play an important role in the cellular response to environmental stress and exert a cytoprotective effect. Especially HSP70 is an effective inhibitor of apoptosis, suggesting a role of HSP70 in carcinogenesis and tumor progression. To explore the relevance of HSP70 in renal cell carcinomas (RCCs), we analyzed nuclear and cytoplasmic HSP70 protein expression in formalin-fixed tissue from 145 clear cell RCCs by immunohistochemistry as well as Western blot analysis.

Nuclear HSP70 expression was found in all RCCs and 75% of the tumors also exhibited a cytoplasmic HSP70 staining. Importantly, RCCs showed significantly reduced cytoplasmic (p=0.001) and combined nuclear/cytoplasmic (p=0.0022) HSP70 expression when compared with their cells of origin. A significant (p=0.0176) decrease of nuclear HSP70 expression became evident from well to poorly differentiated clear cell RCCs. Quite similarly, a trend (p=0.0558) for reduced combined nuclear/cytoplasmic HSP70 expression was shown from early (pT1) to advanced (pT3) tumor stages. Nevertheless, no correlation between HSP70 expression and patients´ survival became evident.

In conclusion, our investigation demonstrates a significant decrease of antiapoptotic HSP70 protein expression during carcinogenesis and during progression from well (G1) to poorly (G3) differentiated clear cell RCCs. Our results suggest that HSP70-mediated inhibition of apoptosis seems to be of minor importance for carcinogenesis and tumor progression in RCCs.

Key words: HSP70-expression, Renal cell carcinoma, Apoptosis, Carcinogenesis, Tumor progression

Offprint requests to: Uwe Ramp, MD, Institute of Pathology, Heinrich-Heine University Hospital, Moorenstr. 5, D-40225 Duesseldorf, Germany. e-mail: Ramp@med.uni-duesseldorf.de

Introduction

Heat shock proteins (HSPs) constitute a highly conserved group of proteins found in nearly all organisms and which are classified by their molecular weights, ranging from 15 to 90 kD. Heat shock proteins are induced in response to sublethal environmental stress, including heat, drugs, irradiation, heavy metals, oxidants, hypoxia and infection (Beck et al., 2000). The cytoprotective effect offered by HSPs against these events is mediated by a process called molecular chaperoning. Molecular chaperones can stabilize unfolded precursor proteins prior to their assembly into multimolecular complexes and prevent inappropriate interactions with other proteins as well as irreversible aggregation of misfolded proteins (Beck et al., 2000).

Especially HSP70 is a prominent cytoprotective protein and several studies provided evidence that the protective effect of HSP70 is - at least partially - mediated by the suppression of apoptosis (Beere and Green, 2001). Thus, HSP70 is able to protect against multiple apoptotic stimuli, including DNA damage, irradiation, serum withdrawal and a variety of anticancer drugs (Beere and Green, 2001). Overexpression of HSP70 in several cell types increased transformation, whereas HSP70 downregulation is sufficient to kill tumor cells or to facilitate the induction of apoptosis (Gurbuxani et al., 2003). The antiapoptotic function of HSP70 involves interactions with the tumor suppressor gene p53 and Bag-1 as well as several components of the apoptotic machinery (Beere et al., 2000; Beere and Green, 2001; Gurbuxani et al., 2003). Thus, HSP70 binds to Apaf-1, thereby preventing the recruitment of procaspase-9 to the apoptosome (Beere et al., 2000). In addition, HSP70 can also inhibit caspase-independent apoptosis by directly interacting with and neutralizing the apoptosis inducing factor (AIF) (Gurbuxani et al., 2003).

Importantly, inhibition of apoptosis by HSP70 also seems to affect tumor progression in vivo, suggesting

that HSP70 might be used as a novel prognostic factor. Thus, recent studies demonstrated a correlation between HSP70 expression and low differentiation, advanced tumor stage or an unfavorable clinical course in carcinomas of the breast (Ciocca et al., 1993; Thanner et al., 2003), stomach (Canöz et al., 2002), colon (Lazaris et al., 1995), urinary bladder (Syrigos et al., 2003) and oral squamous cell carcinomas (Kaur et al., 1998). Nevertheless, the role of HSP70 as a potential prognostic factor is still controversial. Thus, HSP70 expression was observed to correlate with a more favourable prognosis or an early tumor stage in carcinomas of the esophagus (Nakajima et al., 2002; Noguchi et al., 2002), malignant melanomas (Ricaniadis et al., 2001) and nephroblastomas (Efferth et al., 2001), whereas no correlation between HSP70 expression and prognosis became evident in carcinomas of the prostate (Cornford et al., 2000) ovaries (Elpek et al., 2003) and tongue (Ito et al., 1998) as well as in osteosarcomas (Uozaki et al., 2000). In renal cell carcinomas (RCCs) the relevance of HSP70 expression as a potential prognostic factor is still unknown since only a single investigation with a very small tumor number was performed suggesting a correlation with disease-free survival time although no correlation with tumor stage or differentiation was observed (Santarosa et al., 1997).

The aim of the present investigation, therefore, was to explore the relevance of HSP70 protein expression for carcinogenesis, progression and prognosis in a large cohort of RCCs of the clear cell type. Clear cell RCC - which is the most frequent malignant tumor of the kidney - makes up more than 70% of all RCCs and exhibits an extremely variable clinical course, which cannot reliably be predicted (Eble, 1997). Therefore, additional prognostic markers are necessary for a more accurate determination of prognosis.

Materials and methods

Patients

The present retrospective study is based on 170 consecutive patients who underwent potentially curative surgery for RCC of the clear cell type from September 1983 to December 1993 at the University Hospital in Duesseldorf. Potentially curative surgery was defined as the removal of all gross tumor and the demonstration of tumor-negative surgical margins by microscopic examination. No preoperative or postoperative chemotherapy was performed. To eliminate bias due to deaths directly resulting from operation, patients who died within 4 weeks after surgery were excluded from the investigation. Moreover, from some patients no further information was available, leaving 145 patients for the final study. The follow-up ranged from 1 to 158 months after surgery with a median follow-up time of 56 months.

Eighty-seven patients (60%) were male and 58 (40%) were female. The mean age of the patients was 61±10 years (range 34-85 years). Seventeen patients (12%) were between 31 and 50 years old, 53 (37%) were 51-60 years old, 47 (32%) were 61-70 years old, and 28 (19%) patients were older than 71 years.

Pathological review

The surgical specimens from the primary tumors were fixed in 4% buffered formalin and embedded in paraffin. An average of 4 sections per tumor (range 3-9 sections, depending on tumor diameter) was prepared and paraffin sections were routinely stained with hematoxylin and eosin (H&E).

Since the typing, staging and grading of RCCs has markedly changed in the past decade, the paraffin sections of all tumors were re-evaluated by a pathologist. Only RCCs of the clear cell type according to the World Health Organization (Grignon et al., 2004) were included in the investigation. The pT classification and the pN classification were determined according to the criteria proposed by the Union Internationale Contre le Cancer (Wittekind et al., 2002). Accordingly, 41 (28%) of the renal cell carcinomas were in category pT1a, 34 (23%) in category pT1b, 11 (7%) in category pT2, 26 (18%) in category pT3a, 32 (22%) in category pT3b and 1 (1%) in category pT3c. Thirty-six (25%) tumors were categorized as pN0, 6 (4%) as pN1, 1 (1%) as pN2, and 102 (70%) as pNx. No distant metastases had been observed at the time of surgery in any case of our study. The grade of tumor differentiation was determined according to Störkel et al. (1989). Thirty-five (24%) tumors were graded as G1, 80 (55%) as G2, and 30 (21%) as G3.

Immunohistochemistry

For immunohistochemical detection of HSP70 (W27), the monoclonal mouse IgG_{2a} antibody (sc-24, Santa Cruz Biotechnology, USA), was used. 4 µm thick paraffin sections from one to two paraffin blocks representative of the entire tumor were mounted on glass slides coated with poly-L-Lysine and dried overnight at 58 °C. After deparaffination and rehydratation, slides were immersed in 10 mM sodium citrate buffer (pH 6.0) and heated 5 min in a microwave oven at 600 W. After blocking of endogenous peroxidase activity, nonspecific conjugation was blocked with 5% normal horse serum. Endogenous biotin was blocked using an avidin/biotin blocking kit (SP-2001, Vector Laboratories Burlingame, USA). The slides were incubated with the monoclonal HSP70 (W27) antibody (1:4000 dilution) in a moist chamber at 37°C for 60 min. Bound antibody was detected using the avidin-biotin complex (ABC) peroxidase method (ABC Elite Kit, Vector Laboratories, Burlingame, USA). The staining reaction was performed with 3,3'-diaminobenzidine and H_2O_2. For mild counter-staining Mayer's hematoxylin solution was used.

In each HSP70 immunostaining-series, normal urothelial layer and a RCC known to exhibit a strong HSP70 immunoreactivity served as positive control. Negative controls were performed, replacing the primary

antibody by an irrelevant monoclonal mouse antibody.

Evaluation of HSP70 expression

HSP70 expression of each tumor as well as adjacent non-neoplastic renal tissue was examined in two independent immunohistochemical staining reactions and evaluated by a pathologist without knowledge of the clinical outcome.

Since initial experiments revealed nuclear and cytoplasmic HSP70 expression with a marked intratumoral heterogeneity we used a scoring system for the independent assessment of nuclear and cytoplasmic HSP70 expression in RCC and in non-neoplastic renal tissue. Thus, for semi-quantitave analysis, the proportion of nuclear HSP70-positive cells and the corresponding staining intensity were estimated by assessing the whole section. Separately, the proportion of cytoplasmic HSP70-positive cells and the corresponding staining intensity were independently estimated by assessing the whole section.

As shown in Table 1, the intensity of nuclear and cytoplasmic HSP70 staining in cells was determined as 0, indicating no staining, as 1, indicating weak staining intensity, as 2, indicating moderate staining intensity, or 3, indicating strong staining intensity.

Moreover, the percentage of cells with nuclear and cytoplasmic HSP70 positivity was estimated for each staining intensity in steps of 5%. Importantly, the staining intensity as well as the proportion of positive cells was estimated separately for nuclear and cytoplasmic HSP70 expression.

By multiplying the respective proportion of HSP70-positive cells (1-20) with each staining intensity (0-3), a nuclear and a cytoplasmic HSP70 expression score were independently calculated. Thus, a nuclear or cytoplasmic HSP70 expression score of 0 was obtained when no nuclear/cytoplasmic HSP70 staining was observed in the whole section. In contrast, a maximum nuclear/cytoplasmic HSP70 expression score of 60 would be obtained when all cells were stained with strong intensity. Moreover, by addition of the nuclear and cytoplasmic HSP70 expression scores, a *combined* nuclear/cytoplasmic score was calculated.

Because two independent HSP70 immunostaining reactions were performed in each RCC, two nuclear and cytoplasmic HSP70 expression scores were obtained from each tumor and the median was used for statistical analysis.

Furthermore, the RCCs were divided into two groups of tumors showing low levels of HSP70 expression, i.e an expression score ≤ the median expression score of all tumors, or high levels of HSP70 expression, i.e an expression score > the median expression score of all tumors.

Statistical analysis

The correlation between nuclear and/or cytoplasmic HSP70 expression scores and other prognostic parameters was statistically analyzed by means of the Mantel-Haenszel Chi-Square test. Survival rates were calculated by the Kaplan-Meier method for analysis of censored data. The statistical significance of differences in survival was analyzed by means of the log-rank test, and the prognostic significance of parameters in multiparametric analysis by means of a Cox regression analysis. P-values < 0.05 were considered statistically significant.

Western blot analysis

The expression level of HSP70 was also analyzed by Western blot in frozen tissue samples of four arbitrarily selected clear cell RCCs of different histological grades (two well differentiated and two poorly differentiated RCCs). Tumor tissue was lysed in a buffer containing 100 mM NaCl, 10 mM Tris-HCl (pH 7.6), 1 mM EDTA (pH 8.0), 1 µg/ml aprotinin, 100 µg/ml phenyl-methylsulfonyl fluoride and 1% NP40. After centrifugation, equal amounts of protein were analyzed by SDS-PAGE and the expression level of HSP70 was determined by immunoblotting with the monoclonal mouse IgG2a antibody (sc-24, clone W27, Santa Cruz Biotechnology, Santa Cruz, USA; dilution 1:200). For detection, the ECL detection system (Fa. Amersham, Germany) was used according to the manufacturer´s instructions.

Equal loading of the gels was confirmed both by Panceau-red staining of the filters and by re-incubation of the filters with a monoclonal antibody for ß-actin (clone AC-15, Fa. Sigma, Germany).

Results

HSP70 expression in non-neoplastic kidney

In non-neoplastic kidney tissue, a heterogenously distributed weak to moderate nuclear HSP70 immunostaining was found in less than 50% of the proximal and distal tubule epithelia (Fig. 1a), corresponding to a median expression score of 10. Moreover, a weak cytoplasmic HSP70 expression was present in usually more than 70% of the proximal and distal tubule epithelia (Fig. 1a), corresponding to a median expression score of 8. The resulting combined nuclear/cytoplasmic HSP70 expression score of the tubule epithelia, therefore, was 18. A moderate nuclear

Table 1. Staining intensities and percentage of positive cells.

Staining intensity	Percentage of positive cells
0 = no	< 5% = 1
1 = weak	5≤ 10% = 2
2 = moderate	10≤ 15% = 3
3 = strong	15≤ 20% = 4
	et cetera
	95-100% = 20

HSP70 expression could also be detected in the capillary tufts of the glomeruli and the Bowman´s capsule (Fig. 1a). Furthermore, the lower 2/3 of the urothel layer exhibited a moderate to strong nuclear and cytoplasmic HSP70 immunostaining (Fig. 1b).

Nuclear and cytoplasmic HSP70 expression in clear cell renal carcinomas

All (n=145) investigated renal cell carcinomas (RCCs) of the clear cell type exhibited nuclear HSP70 expression, whereas only 75% (n=109) of the RCCs demonstated cytoplasmic HSP70 staining.

Low (score ≤ median) *nuclear* HSP70 expression was observed in 52% (n=75) of the RCCs (Fig. 2a), whereas 48% (n=70) of the tumors exhibited a high (score > median) nuclear HSP70 immunoreactivity (Fig. 2b). Out of the 75% RCCs (n=109) demonstrating a positive cytoplasmic HSP70 expression, 25% (n=37) exhibited a low cytoplasmic expression (Fig. 2c), whereas 50% (n=72) of the tumors exhibited a high cytoplasmic HSP70 immunoreactivity (Fig. 2d).

In RCCs, HSP70 immunoreactivity was predominantly located in the nucleus with a median nuclear expression score of 8, whereas the median cytoplasmic expression score and the combined nuclear/cytoplasmic expression score was only 1 and 9, respectively. Clear cell RCCs, therefore, exhibited a significantly reduced cytoplasmic (p=0.001) as well as combined nuclear/cytoplasmic (p=0.0022) HSP70 expression, when compared with their cells of origin, i.e. the tubule epithelia.

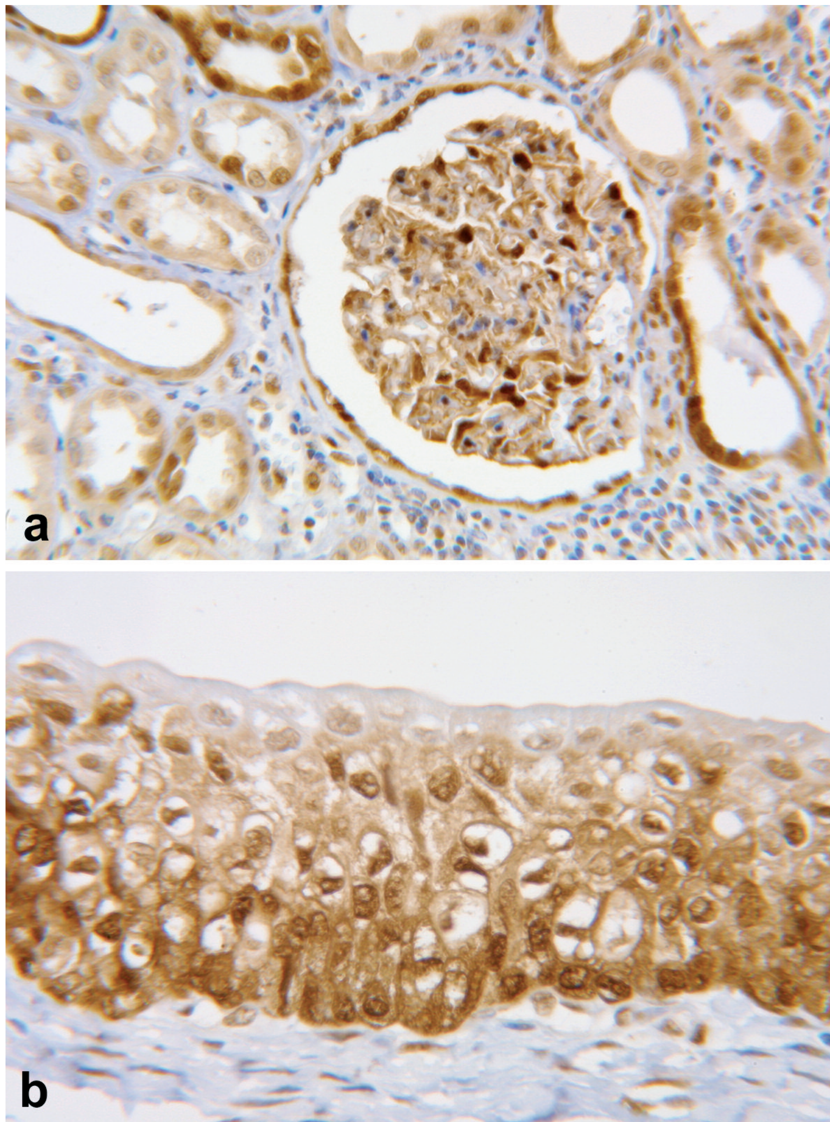

Fig. 1. Immunohistochemical expression of HSP70 protein expression in normal kidney and normal urothel. **a.** Weak to moderate nuclear HSP70 immunostaining in less than 50% of the tubule epithelia and a weak cytoplasmic HSP70 expression in most tubule epithelia. **b.** Moderate to strong nuclear and cytoplasmic HSP70 immunostaining in the lower 2/3 of the urothel layer.

Correlation of nuclear HSP70 expression with tumor grade

A significant correlation (p=0.0176) became evident between nuclear HSP70 expression and tumor differentiation (Table 2). Thus, 70% of the poorly differentiated (G3) RCCs exhibited only a low nuclear HSP70 expression (score≤ median). In contrast, a high nuclear HSP70 immunoreactivity (score > median) could be found in 60% of the highly differentiated (G1) tumors. Accordingly, the median of the nuclear HSP70 expression scores decreased from 9.3 in well differentiated (G1) RCCs to 8.3 in G2 tumors and 7.4 in poorly differentiated (G3) tumors.

Moreover, but not significant, advanced tumor stages (pT2 and pT3) had a marginally lower nuclear HSP70 expression (Table 2).

No correlation could be demonstrated between nuclear HSP70 expression and the pN category, patients'age at time of diagnosis or patients'sex (Table 2).

No correlation of cytoplasmic HSP70 expression with clinico-pathological parameters

No correlation became evident between cytoplasmic

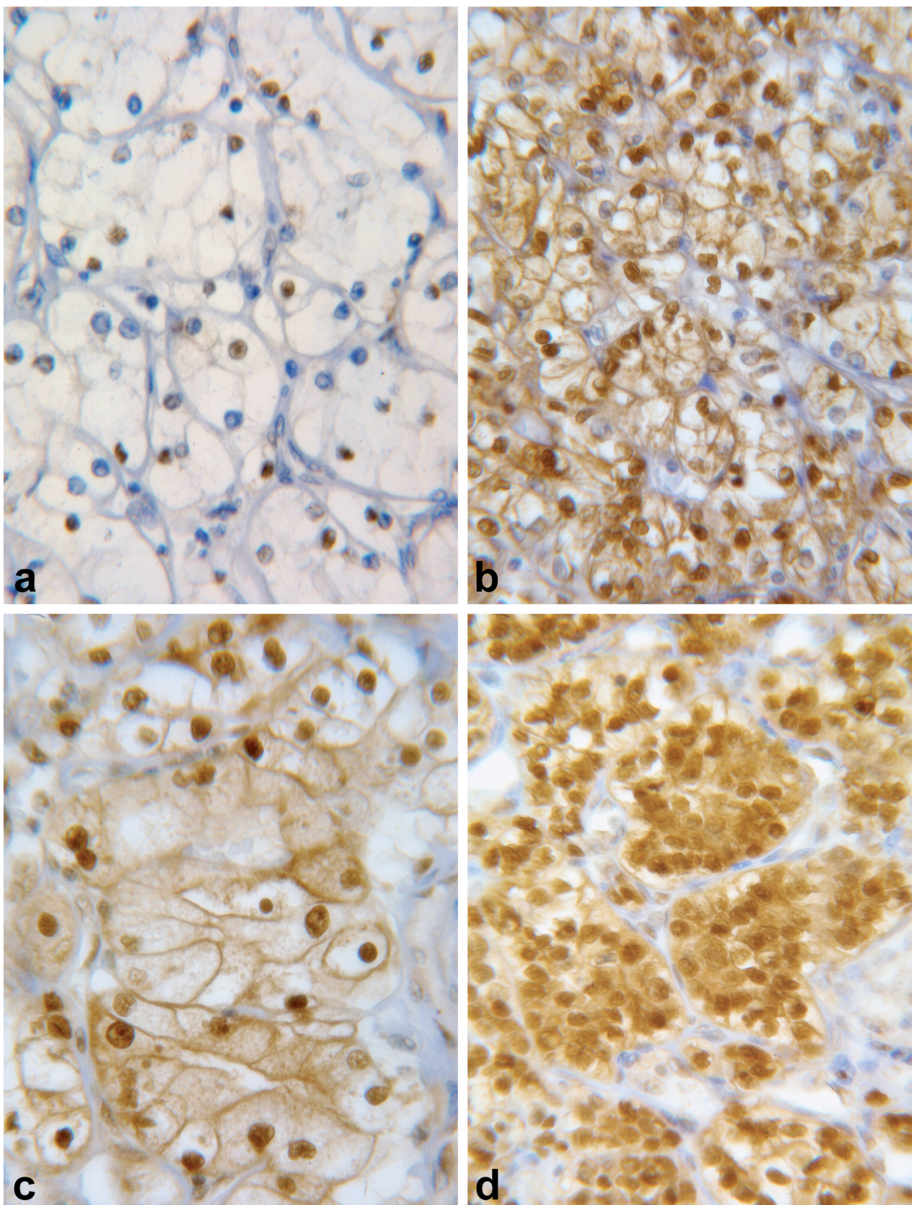

Fig. 2. Immunohistochemical expression of nuclear and/or cytoplasmic HSP70 protein in clear cell RCCs. **a.** Heterogenously distributed weak nuclear HSP70-immunostaining in some tumor cells. **b.** Moderate to strong nuclear HSP70 expression in the majority of tumor cells. **c.** Heterogenously distributed weak cytoplasmic HSP70 expression in some tumor cells in combination with a moderate nuclear HSP70 expression. **d.** Moderate to strong nuclear and cytoplasmic HSP70 expression in nearly all tumor cells.

HSP70 expression and tumor grade, tumor stage, the pN category, patients'age at time of diagnosis or patients'sex (Table 3).

Relation of combined nuclear/cytoplasmic HSP70 expression with tumor stage

Our investigation demonstrated a trend (p=0.0558) towards reduced combined nuclear/cytoplasmic HSP70 expression and advanced tumor stages (Table 4). Thus, the advanced pT2 and pT3 stages were dominated by tumors with low combined HSP70 expression, whereas 61% of the early stage pT1a RCCs exhibited a high combined HSP70 expression.

Although not significant, 60% of the poorly differentiated (G3) RCCs had low combined nuclear/cytoplasmic HSP70 expression, whereas 60% of the well differentiated (G1) tumors exhibited a high combined HSP70 immunoreactivity (Table 4).

No correlation could be demonstrated between combined nuclear/cytoplasmic HSP70 expression and the pN category, patients'age at time of diagnosis or patients'sex (Table 4).

Western blot analysis of HSP70 expression in frozen RCC tissue samples

Although a monoclonal HSP70-antibody was used in our immunohistochemical investigation, we also

Table 2. Nuclear HSP70 expression score (% of tumors).

	Low (Score ≤ median)	High (Score > median)	p-value
Grading			
G1 (n=35)	40	60	0.0176
G2 (n=80)	50	50	
G3 (n=30)	70	30	
Staging			
pT1a (n=41)	41	59	0.1349
pT1b (n=34)	50	50	
pT2 (n=11)	73	27	
pT3 (n=59)	56	44	
pN category			
N-negative (n=36)	58	42	0.5216
N-positive (n=7)	71	29	
Patients'age (years)			
≤ 60 (n=70)	51	49	0.9453
> 60 (n=75)	52	48	
Patients'sex			
m (n=87)	54	46	0.4990
w (n=58)	48	52	

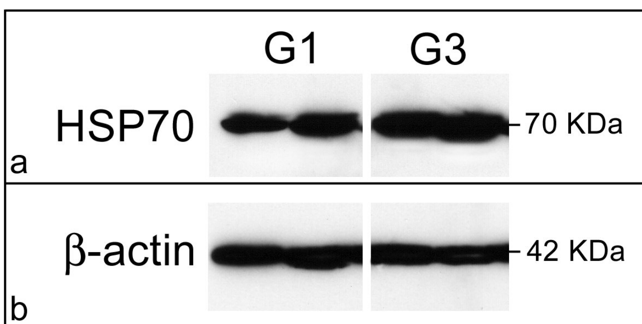

Fig. 3. a. Western blot analysis demonstrating one HSP70-signal of the expected size (70 kDa) in all primary clear cell RCCs. **b.** Equal protein loading of the gels was confirmed by re-incubation of the filters with an antibody against ß-actin.

Table 3. Cytoplasmic HSP70 expression score (% of tumors).

	Low (Score ≤ median)	High (Score > median)	p-value
Grading			
G1 (n=35)	51	49	0.5388
G2 (n=80)	53	48	
G3 (n=30)	43	57	
Staging			
pT1a (n=41)	49	51	0.7257
pT1b (n=34)	44	56	
pT2 (n=11)	82	18	
pT3 (n=59)	49	51	
pN category			
N-negative (n=36)	58	42	0.2322
N-positive (n=7)	57	43	
Patients'age (years)			
≤ 60 (n=70)	53	47	0.5602
> 60 (n=75)	48	52	
Patients'sex			
m (n=87)	56	44	0.0789
w (n=58)	41	59	

Table 4. Combined nuclear and cytoplasmic HSP70 expression score (% of tumors).

	Low (Score ≤ median)	High (Score > median)	p-value
Grading			
G1 (n=35)	40	60	0.1067
G2 (n=80)	51	49	
G3 (n=30)	60	40	
Staging			
pT1a (n=41)	39	61	0.0558
pT1b (n=34)	44	56	
pT2 (n=11)	82	18	
pT3 (n=59)	56	44	
pN category			
N-negative (n=36)	56	44	0.4414
N-positive (n=7)	71	29	
Patients'age (years)			
≤ 60 (n=70)	50	50	0.9363
> 60 (n=75)	51	49	
Patient'sex			
m (n=87)	54	46	0.2796
w (n=58)	45	55	

performed Western blot analysis to exclude non-specific cross reactions with other proteins. For this purpose, frozen tissue samples of 4 arbitrarily selected primary clear cell RCCs of different histological grades were analyzed by Western blot. As can be seen in Fig. 3, hybridization of these tumor tissue samples with the HSP70-antibody resulted in one major band of the expected size (70 kDa).

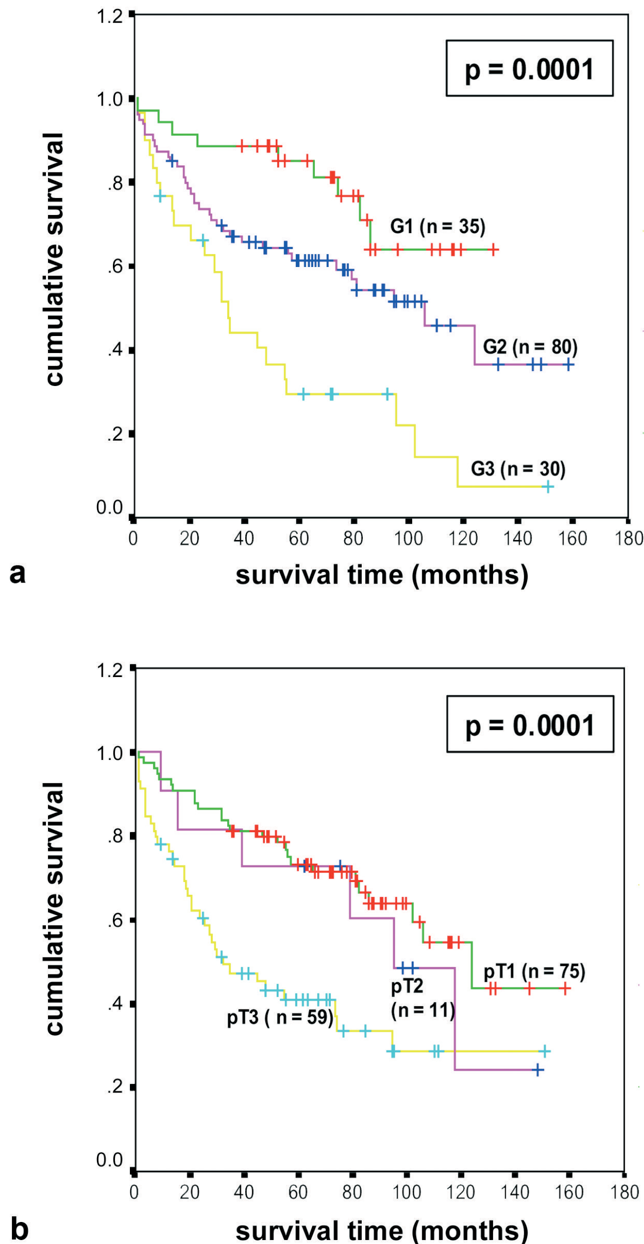

Fig. 4. a. Survival rates of 145 clear cell RCC cancer patients in relation to tumor grade (G1: n=35; G2: n=80; G3: n=30). Log-rank test: p=0.0001. **b.** Survival rates of 145 clear cell RCC cancer patients in relation to tumor stage according to UICC (Wittekind et al., 2002) (pT1a: n=41; pT1b: n=34; pT2: n=11; pT3: n=59). Log-rank test: p=0.0001.

Survival analysis

The median postoperative survival time for all patients was 55.5 months. Since tumor grade and stage are major prognostic parameters in RCC (Srigley et al., 1997), we first analyzed their impact on postoperative survival in our tumor series. As expected, log-rank test revealed a significant inverse correlation between survival and tumor grading (p=0.0001; Fig. 4a) or staging (p=0.0001; Fig. 4b).

Analysis based on the log-rank test, however, revealed no significant correlation between nuclear, cytoplasmic or combined nuclear/cytoplasmic HSP70 expression and survival time of RCC patients (data not shown).

Discussion

Our investigation demonstrated significantly reduced cytoplasmic as well as combined nuclear/cytoplasmic HSP70 expression in clear cell RCCs when compared with their cells of origin, i.e. the tubule epithelia. The high HSP70 expression in tubule epithelia of normal kidney might be explained by its major functional importance providing universal cytoprotection against naturally occuring ischemia, hypotonic stress, urea, high concentrations of inorganic salts and heat (Beck et al., 2000). This situation in tubule epithelial cells, however, contrasts with observations in stomach, colon and urinary bladder, where HSP70 expression was shown to increase in carcinomas when compared with corresponding non-neoplastic tissue (Isomoto et al., 2003; Kanazawa et al., 2003; Syrigos et al., 2003).

Associated with its cytoprotective function, HSP70 is a potent inhibitor of apoptosis thought to be involved in carcinogenesis and tumor progression. The relevance, however, of HSP70 as a prognostic factor in malignant tumors is still not clear with controversial results reported in the literature. Thus, HSP70 expression was observed to correlate with low differentiation, advanced tumor stage or worse prognosis in breast carcinomas (Ciocca et al., 1993; Thanner et al., 2003) colo-rectal carcinomas (Lazaris et al., 1995), gastric cancer (Canöz et al., 2002), carcinomas of the urinary bladder (Syrigos et al., 2003) and oral squamous cell carcinomas (Kaur et al., 1998). In contrast, HSP70 expression was shown to correlate with a more favourable prognosis or an early tumor stage in esophagus carcinomas (Nakajima et al., 2002; Noguchi et al., 2002), malignant melanomas (Ricaniadis et al., 2001) and nephroblastomas (Efferth et al., 2001). In pancreatic adenocarcinomas, HSP70 expression could even be used as an independent factor of a more favourable prognosis (Sagol et al., 2002). Nevertheless, several investigations failed to demonstrate any relevance of HSP70 expression for tumor progression or prognosis in carcinomas of the prostate (Cornford et al., 2000) ovaries (Elpek et al., 2003) and tongue (Ito et al., 1998) as well as in osteosarcomas (Uozaki et al., 2000). In RCC, the relevance of HSP70 expression as a potential prognostic

factor is still unknown since only a single investigation with a very small tumor number and without discriminating between nuclear and cytoplasmic protein expression was performed up to now (Santarosa et al., 1997). In this investigation, a positive correlation with disease-free survival time was suggested, although no correlation with tumor stage or differentiation was observed (Santarosa et al., 1997).

Our data in a large cohort of 145 clear cell RCCs show that the nuclear expression levels of HSP70 protein significantly decreased in poorly differentiated (G3) RCCs. Thus, the median of the nuclear HSP70 protein expression scores decreased from 9.3 in well differentiated (G1) RCCs to 8.3 in G2 tumors and to 7.4 in poorly differentiated (G3) tumors. Similarly, although only a trend, the combined nuclear/cytoplasmic HSP70 protein expression decreased with advanced tumor stages. In summary, our results clearly demonstrate that nuclear HSP70 protein expression markedly decreases in poorly differentiated (G3) clear cell RCCs. Partially similar results were observed in carcinomas of the cervix (Park et al., 1999) and esophagus (Nakajima et al., 2002), gastric cancer (Isomoto et al., 2003) as well as non-small cell lung carcinomas (Malusecka et al., 2001) where tumors of early stage or high differentiation expressed higher HSP70 protein levels. Nevertheless, since no significant correlation between nuclear, cytoplasmic or combined nuclear/cytoplasmic HSP70 expression and survival time of RCC patients became evident, our results also show that HSP70 expression is not suitable as a prognostic marker in clear cell RCCs.

The relevance of intracellular HSP70 protein localisation has been addressed in only a few studies which suggested that HSP70 is mostly cytoplasmic in unstressed cells and translocates to the nucleus after exposure to different forms of stress including heat, anticancer drugs and hypoxia (Vargas-Roig et al., 1998). In our investigation, HSP70 protein was predominantly located in the nucleus of the RCC cells with a median nuclear expression score of 8 whereas the median cytoplasmic expression score was only 1. Interestingly, the median cytoplasmic expression score of non-neoplastic tubule epithelia was 8, thereby indicating higher levels of various cellular stresses in tumor cells.

The underlying mechanisms of decreased nuclear HSP70 protein expression in poorly differentiated (G3) clear cell RCCs are not known yet. Since the majority of tubule epithelia, which are the cells of origin for clear cell RCCs, express considerable amounts of HSP70 protein, it is reasonable to assume a major functional importance of HSP70-mediated cytoprotection and apoptosis-inhibition in non-neoplastic tubule epithelial cells. Since resistance to apoptosis provides a survival advantage also for tumor cells, expression of HSP70 might – at least in part – be preserved in early stage and low grade RCCs. During progression in advanced stage and high nuclear grade RCCs, however, the tumor cells have acquired additional genetic alterations, which might provide more effective mechanisms of apoptosis-inhibition and survival advantage. Thus, we could demonstrate severe disturbances of multiple apoptosis-regulating pathways in RCCs, like CD95 (Ramp et al., 2000), TRAIL (Krieg et al., 2003; Ramp et al., 2003) and XIAP (Ramp et al., 2004; Yan et al., 2004) resulting in alternative mechanims of effective apoptosis-resistance in tumor cells. In consequence, the dependance of RCCs especially to HSP70-mediated apoptosis-inhibition decreases in poorly differentiated RCCs and during tumor progression obviously allowing reduced expression of HSP70 protein in advanced stage and high grade RCCs.

In conclusion, our investigation demonstrates a significant decrease of antiapoptotic HSP70 protein expression during carcinogenesis and during progression from well (G1) to poorly (G3) differentiated clear cell RCCs. Moreover, no correlation between HSP70 expression and prognosis of RCC patients became evident. Our results suggest that HSP70-mediated inhibition of apoptosis seems to be of minor importance for carcinogenesis and tumor progression in clear cell RCCs.

Acknowledgements. We express appreciation to Mrs. S. Schneeloch, Mrs. C. Golmina, Mrs. M. Bellack, Mrs. H. Auweiler and Mr. M. Ringler for excellent technical assistance.

References

Beck F.X., Neuhofer W. and Müller E. (2000). Molecular chaperones in the kidney: distribution, putative roles, and regulation. Am. J. Physiol. Renal. Physiol. 279, 203-215.

Beere H.M., Wolf B.B., Cain K., Mosser D.D., Mahboubi A., Kuwana T., Tailor P., Morimoto R.I., Cohen G.M. and Green D.R. (2000). Heat-shock protein 70 inhibits apoptosis by preventing recruitment of procaspase-9 to the Apaf-1 apoptosome. Nature Cell Biol. 2, 469-475.

Beere H.M. and Green D.R. (2001). Stress management – heat shock protein-70 and the regulation of apoptosis. Trends Cell Biol. 11, 6-10.

Canöz Ö., Belenli O. and Patiroglu T.E. (2002). General features of gastric carcinomas and comparison of Hsp70 and NK cell immunoreactivity with prognostic factors. Pathol. Oncol. Res. 8, 262-269.

Ciocca D.R., Clark G.M., Tandon A.K., Fuqua S.A., Welch W.J. and McGuire W.L. (1993). Heat shock protein hsp70 in patients with axillary lymph node-negative breast cancer: prognostic implications. J. Natl. Cancer Inst. 85, 570-574.

Cornford P.A., Dodson A.R., Parsons K.F., Desmond A.D., Woolfenden A., Fordham M., Neoptolemos J.P., Ke Y. and Foster C.S. (2000). Heat shock protein expression independently predicts clinical outcome in prostate cancer. Cancer Res. 60, 7099-7105.

Eble J.N. (1997). Neoplasms of the kidney. In: Urologic surgical pathology. Bostwick D.G. and Eble J.N. (eds). Mosby. St. Louis. pp 83-147.

Efferth T., Schulten H.G., Thelen P., Bode M.E., Beniers A.J.M.C., Granzen B., Ringert R.H., Mertens R., Gefeller O., Jakse G. and Fuzesi L. (2001). Differential expression of the heat shock protein70

in the histological compartments of nephroblastomas. Anticancer Res. 21, 2915-2920.

Elpek G.O., Karaveli S., Simsek T., Keles N. and Aksoy N.H. (2003). Expression of heat shock proteins hsp27, hsp70 and hsp90 in malignant epithelial tumour of the ovaries. APMIS 111, 523-530.

Grignon D.J., Eble J.N., Bonsib S.M. and Moch H. (2004). Clear cell renal cell carcinoma, In: Pathology & Genetics, Tumours of the urinary system and male genital organs. Eble J.N., Sauter G., Epstein J.I. and Sesterhenn I.A. (eds). IARC Press. Lyon. pp 23-25.

Gurbuxani S., Schmitt E., Cande C., Parcellier A., Hammann A., Daugas E., Kouranit I., Spahr C., Pance A., Kroemer G. and Garrido C. (2003). Heat shock protein 70 binding inhibits the nuclear import of apoptosis-inducing factor. Oncogene 22, 6669-6678.

Isomoto H., Oka M., Yano Y., Kanazawa Y., Soda H., Terada R., Yasutake T., Nakayama T., Shikuwa S., Takeshima F., Udono H., Murata I., Ohtsuka K. and Kohno S. (2003). Expression of heat shock protein (Hsp) 70 and Hsp 40 in gastric cancer. Cancer Lett. 198, 219-228.

Ito T., Kawabe R., Kurasono Y., Hara M., Kitamura H., Fujita K. and Kanisawa M. (1998). Expression of heat shock proteins in squamous cell carcinoma of the tongue: an immunohistochemical study. J. Oral Pathol. Med. 27, 18-22.

Kanazawa Y., Isomoto H., Oka M., Yano Y., Soda H., Shikuwa S., Takeshima F., Omagari K., Mizuta Y., Murase K., Nakagoe T., Ohtsuka K. and Kohno S. (2003). Expression of heat shock protein (Hsp) 70 and Hsp 40 in colorectal cancer. Med. Oncol. 20, 157-164.

Kaur J., Srivastava A. and Ralhan R. (1998). Expression of 70-kDa heat shock protein in oral lesions: marker of biological stress or pathogenicity. Oral Oncol. 34, 496-501.

Krieg A., Krieg T., Wenzel M., Schmitt M., Ramp U., Fang B., Gabbert H.E., Gerharz C.D. and Mahotka C. (2003). TRAIL-ß and TRAIL-γ. Two novel splice variants of the human TNF-related apoptosis inducing ligand (TRAIL) without apoptotic potential. Br. J. Cancer 88, 918-927.

Lazaris A.C., Theodoropoulos G.E., Davaris P.S., Panoussopoulos D., Nakopoulou L., Kittas C. and Golematis B.C. (1995). Heat shock protein 70 and HLA-DR molecules tissue expression. Prognostic implications in colorectal cancer. Dis. Colon Rectum 38, 739-745.

Malusecka E., Zborek A., Krzyzowska-Gruca S. and Krawczyk Z. (2001). Expression of heat shock proteins HSP70 and HSP27 in primary non-small cell lung carcinomas. An immunohistochemical study. Anticancer Res. 21, 1015-1022.

Nakajima M., Kuwano H., Miyazaki T., Masuda N. and Kato H. (2002). Significant correlation between expression of heat shock protein 27, 70 and lymphocyte infiltration in esophageal squamous cell carcinoma. Cancer Lett. 178, 99-106.

Noguchi T., Takeno S., Shibata T., Uchida Y., Yogoyama S. and Müller W. (2002). Expression of heat shock protein 70 in grossly resected esophageal squamous cell carcinoma. Ann. Thorac. Surg. 74, 222-226.

Park C.S., Joo I.S., Song S.Y., Kim D.S., Bae D.S. and Lee J.H. (1999). An immunohistological analysis of heat shock protein 70, p53, and estrogen receptor status in carcinoma of the uterine cervix. Gynecol. Oncol. 74, 53-60.

Ramp U., Déjosez M., Mahotka C., Czarnotta B., Kalinski T., Wenzel M., Müller M., Krammer P., Gabbert H.E. and Gerharz C.D. (2000). Deficient activation of CD95 (APO-1/Fas)-mediated apoptosis: a potential factor of multidrug resistance in human renal cell carcinoma. Br. J. Cancer 82, 1851-1859.

Ramp U., Caliskan E., Mahotka C., Krieg A., Heikaus S., Gabbert H.E. and Gerharz C.D. (2003). Apoptosis-induction in renal cell carcinoma by TRAIL and γ-radiation is impaired by deficient caspase-9 cleavage. Br. J. Cancer 88, 1800-1807.

Ramp U., Krieg T., Caliskan E., Mahotka C., Ebert T., Willers R., Gabbert H.E. and Gerharz C.D. (2004). XIAP expression is an independent prognostic marker in clear-cell renal carcinomas. Hum. Pathol. 35, 1022-1028.

Ricaniadis N., Kataki A., Agnantis N., Androulakis G. and Karakousis C.P. (2001). Long-term prognostic significance of HSP-70, c-myc and HLA-DR expression in patients with malignant melanoma. EJSO 27, 88-93.

Sagol Ö., Tuna B., Coker A., Karademir S., Obuz F., Astarcioglu H., Küpelioglu A., Astarcioglu I. and Topalak Ö. (2002). Immunohistochemical detection of p52 protein and heat shock protein-70 in pancreatic adenocarcinomas. Relationship with disease extent and patient survival. Pathol. Res. Pract. 198, 77-84.

Santarosa M., Favaro D., Quaia M. and Galligioni E. (1997). Expression of heat shock protein 72 in renal cell carcinoma: possible role and prognostic implications in cancer patients. Eur. J. Cancer 33, 873-877.

Srigley J.R., Hutter R.V., Gelb A.B., Henson D.E., Kenney G., King B.F., Raziuddin S. and Pisansky T.M. (1997). Current prognostic factors - renal cell carcinoma: Workgroup No. 4. Union Internationale Contre le Cancer (UICC) and the American Joint Committee on Cancer (AJCC). Cancer 80, 994-996.

Störkel S., Thoenes W., Jacobi G.H. and Lippold R. (1989). Prognostic parameters in renal cell carcinoma – a new approach. Eur. Urol. 16, 416-422.

Syrigos K.N., Harrington K.J., Karayiannakis A.J., Sekara E., Chatziyianni E., Syrigou E.I. and Waxman J. (2003). Clinical significance of heat shock protein-70 expression in bladder cancer. Urology 61, 677-680.

Thanner F., Sütterlin M.W., Kapp M., Rieger L., Kristen P., Dietl J., Gassel A.M. and Müller T. (2003). Heat-shock protein 70 as a prognostic marker in node-negative breast cancer. Anticancer Res. 23, 1057-1062.

Uozaki H., Ishida T., Kakiuchi C., Horiuchi H., Gotoh T., Iijima T., Imamura T. and Machinami R. (2000). Expression of heat shock proteins in osteosarcoma and its relationship to prognosis. Pathol. Res. Pract. 196, 665-673.

Vargas-Roig L.M., Gago F.E., Tello O., Aznar J.C. and Ciocca D.R. (1998). Heat shock protein expression and drug resistance in breast cancer patients treated with induction chemotherapy. Int. J. Cancer 79, 468-475.

Wittekind Ch., Meyer H.J. and Bootz F. (2002). TNM Klassifikation maligner Tumoren. International Union Against Cancer (UICC). Springer. Berlin, Heidelberg, New York. pp 181-183.

Yan Y., Mahotka C., Heikaus S., Shibata T., Wethkamp N., Liebmann J., Suschek C.V., Guo Y., Gabbert H.E., Gerharz C.D. and Ramp U. (2004). Disturbed balance of expression between XIAP and Smac/DIABLO during tumor progression in renal cell carcinomas. Br. J. Cancer 91, 1349-1357.

Accepted April 13, 2007